KARLOVA UNIVERZITA V PRAZE
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KATEDRA FARMAKOLOGIE A TOXIKOLOGIE

Diplomová práce

Účinek kyseliny nikotinové na aktivaci makrofágů při iktu

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INSTITUTU FARMAKOLOGIE
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Heidelberg & Hradec Králové, 2011 Barbora Pokorná

CHARLES UNIVERSITY IN PRAGUE
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Diploma Thesis

Effect of Nicotinic Acid on infiltration of macrophages in the ischemic brain

PERFORMED AT
INSTITUTE OF PHARMACOLOGY
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I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, all of them are given in the list of used literature and they are quoted in text regularly. This work was not used to obtain any other title.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány. Tato práce nebyla využita k získání jiného či stejného titulu.

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Barbora Pokorná

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ABSTRACT

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Title of diploma thesis: Effect of Nicotinic Acid on infiltration of macrophages in the ischemic brain

Stroke is one of the leading causes of death worldwide and its effective protective treatment is still missing.

Nicotinic acid has been a widely used substance to modify lipid profiles for many years and it successfully prevents clinical cardiovascular diseases. Recently explored receptor, GPR109A, causes its effect and it was found to be expressed in spleen, adipose, and immune cells including macrophages. The aim of this work is to investigate the effect of nicotinic acid on the infarct size during cerebral ischemia and to determine the role of macrophages in its effect.

We compared two groups in our experiments, transgenic CD11bDTR mice and control wild type (WT) mice. Both groups were treated with diphtheria toxin (DT), then a middle cerebral artery occlusion was performed and afterwards mice were treated with either nicotinic acid or vehicle.

We have found depletion of macrophages in CD11bDTR group induced by DT treatment and depletion of macrophages in WT group treated with nicotinic acid. We also found significant difference in infarct volume in the wild type mice that are treated with nicotinic acid than their corresponding control.

According to our results, nicotinic acid seems to have a protective effect on cerebral ischemia.

ABSTRAKT

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Iktus se řadí k jedné z nejčastějších příčin úmrtí na světě a jeho efektivní terapie s protektivním účinkem stále chybí.
Nikotinová kyselina se široce terapeuticky používá pro ovlivnění lipidového spektra a jsou také známy její kardiovaskulárně preventivní účinky. Nedávno objevený receptor, GPR109A, pravděpodobně zprostředkuje její účinek a nachází se ve slezině, tukové tkáni a imunitních buňkách včetně makrofágů.
Cílem této práce je zjistit, jaký efekt má nikotinová kyselina na rozsah iktu a jakou roli hrají makrofágy v jejím účinku.
Při experimentech jsme srovnávali dvě skupiny myší, geneticky upravené myši CD11bDTR a kontrolní *wildtype* skupinu. Obě skupiny byly léčeny difterotoxinem, následně byl operativně vyvolán iktus a polovina myší byla posléze léčena kyselinou nikotinovou a polovina pouze vehikulem.
Zjištěným výsledkem byla deplece makrofágů ve skupině geneticky modifikovaných CD11bDTR myší vyvolaná podáním difterotoxinu. V kontrolní skupině wildtype myší vyvolalo podání kyseliny nikotinové rovněž depleci makrofágů. Zároveň byl v této skupině prokázán nižší rozsah iktu oproti ostatním skupinám. Dle našich výsledků se nikotinová kyselina zdá být nadějnou látkou s protektivním účinkem při iktu.

ABBREVIATIONS LIST

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	antigen presenting cell
ATP	adenosine triphosphate
BBB	blood brain barrier
BSA	bovine serum albumine
CAD	caspase-activated deoxyribonuclease
cAMP	cyclic adenosine monophosphate
CINC	cytokine-induced neutrophil chemoattractant
CNS	central nervous system
CVA	cerebrovascular accident
DAPI	4‘,6‘- diamidino-2-phenylindole
DT	diphtheria toxin
FACS	Fluorescence-activated cell sorting
FADD	Fas- associated death domain
FST	Fine Science Tools
GE	Germany
GPCR	G-protein-coupled receptor
HDL	high-density lipoprotein
HSC	haematopoietic stem cell
ICAM	intercellular adhesion molecule
IFN γ	interferon γ
IL	interleukin
LDL	low-density lipoprotein
MCAO	middle cerebral artery occlusion
MCP	monocyte chemoattractant protein
MF	macrophage/macrophages
MyD88	myeloid differentiation primary-response gene 88
NA	nicotinic acid
NAD+	β-nicotinamide adenine dinucleotide
NK	natural killer
NMDA	N-methyl-D-aspartate

NO	nitric oxide
NOS	nitric-oxide synthase
PARP-1	poly(ADP-ribose)polymerase-1
PG	prostaglandin
PBS	phosphate buffered saline
PBMcs	peripheral blood mononuclear cells
PFA	paraformaldehyde
rt-PA	recombinant tissue plasminogen activator
TGF-β	transforming growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VLDL	very-low-density lipoprotein
WT	wild-type

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1. INTRODUCTION

1.1. STROKE

1.1.1. Definition and epidemiology of stroke

The term stroke, also referred to as cerebrovascular accident (CVA), broadly refers to neurologic symptoms and indicates interrupted blood flow to brain tissue (Nestler Eric 2009). Stroke is one of the leading causes of morbidity and mortality worldwide. It is the third leading cause of death after cardiovascular diseases and cancer in industrialized countries (Thorvaldsen et al. 1995). According to WHO estimates, 15 million people each year suffer strokes and 5 million are left permanently disabled. Stroke accounts for a higher proportion of deaths among women than men (11% vs. 8.4%). Among women, 3 million deaths from stroke occur annually (WHO, 2004).

Consequences of stroke are in enormous medical, economic, and personal costs. Survivors of stroke are often affected by serious long-term disabilities such as paralysis and disruption of higher cognitive functions such as speech. These patients are unable to resume work and other activities, and often need extensive long-term care by healthcare professionals or friends and family (Nestler Eric 2009).

1.1.2. Classification

Stroke occurs upon disruption of blood flow to brain tissue caused by obstruction or hemorrhage and that separates the two primary types of stroke- hemorrhagic and occlusive.

A hemorrhagic stroke is caused by bleeding from a vessel and it usually occurs in selected parts of the brain, including the basal ganglia, cerebellum, brainstem, or cortex. Intracerebral hemorrhage is divided in the intraparenchymal, epidural, subdural, or subarachnoid type (Nestler Eric 2009).

An occlusive stroke, also called cerebral ischemia, occurs when the blood vessel becomes blocked. Blockage can be caused by embolic, atherosclerotic, or thrombotic occlusion of cerebral vessels. Vascular occlusion results in neurologic deficits and in a loss of functions controlled by the affected region (Nestler Eric 2009).
Cerebral ischemia can be differentiated into focal and global cerebral ischemia. Focal cerebral ischemia results in a damage of the same region where a blockage in an artery occurs. However, damage can be reduced by forming collateral blood supply in contrast to global cerebral ischemia. Focal cerebral ischemia is differentiated into transient and permanent cerebral ischemia. In our study a mouse model of permanent cerebral ischemia is used to investigate ischemic stroke (Hossmann 1998).

1.1.3. Aetiology and risk factors

Risk factors for stroke can be classified as modifiable, potentially modifiable and non-modifiable (Sacco 1997).
The risk factors for stroke that can not be changed include age, gender, family history and ethnicity (Sacco 1997).
Modifiable factors that are associated with an increased risk of stroke involve the history of hypertension, current smoking, waist-to-hip ratio, diet risk score (increased risk associated with increased consumption of red meat, organ meats or eggs, fried foods and cooking with lard), lack of physical activity, diabetes mellitus, more than 30 alcoholic drinks per month or binge drinking, psychological stress, other heart problems, and cholesterol (O'Donnell et al.). According to the INTERSTROKE study (O'Donnell et al.), all of these factors are linked with ischaemic stroke. Only hypertension, smoking, waist-to-hip ratio, diet and alcohol are linked to intracerebral haemorrhagic stroke.

1.1.4. Therapy

Successful treatment can be achieved only with appropriate therapy.
Therapy depends on the type of stroke and also on the time after onset of stroke and it always aims to reduce or prevent brain infarction and minimize the long term disability

and stroke-related mortality. Stroke therapy always includes medical treatment and sometimes also surgical treatment. Initial treatment for ischemic stroke involves removing of the blockage and restoring of blood flow, which can be achieved with trombolysis. Recombinant tissue plasminogen activator (rt-PA) is the only licensed drug for intravenous use in highly selected patients within three hours of the event (Grotta and Marler 2007). One of the possible side effects of rt-PA and other thrombolytic drugs used in intra-arterial therapy include serious bleeding in the brain, which can be fatal. Anyhow, thrombolysis is the only instrument of reversing vessel obstruction and inducing reperfusion. Other treatment is widely used as secondary prevention such as antiplatelet medication and anticoagulants to help prevent the formation of blood clots and to reduce the risk for recurrent stroke. The choice of this medical treatment is very individual, long term and does not affect the lysis of the clot. However, therapy that is effective after several hours after onset of cerebral ischemia is still missing.

1.2. PATHOPHYSIOLOGY OF CEREBRAL ISCHEMIA

The brain represents only 2% of the body's total mass but it uses approximately 20% of the body's oxygen supply, and blood flow to the brain uses about 15% of total cardiac output (Nestler Eric 2009). In the acute phase of focal cerebral ischemia, brain vessel becomes occluded. It results in insufficient oxygen and glucose delivery and serious neuronal damage can occur within minutes of an ischemic event (Nestler Eric 2009).

Complex series of cellular and molecular events is rapidly set in cascade that leads to cell death: excitotoxicity and ionic imbalance, periinfarct depolarization, inflammation and programmed cell death (apoptosis) (Dirnagl et al. 1999). These processes are overlapping and each of these events occurs in a distinct time frame from the cerebral ischemia onset (Fig.1). The relative contributions of each process are believed to vary significantly in relation to the level of cerebral blood flow.

In the core of an infarct where blood flow is most severely restricted, the main process is energy failure and rapid necrotic cell death. This core is surrounded by reversibly injured area called ischemic penumbra, where collateral blood flow can evade the full effects of the stroke (Liu et al.).

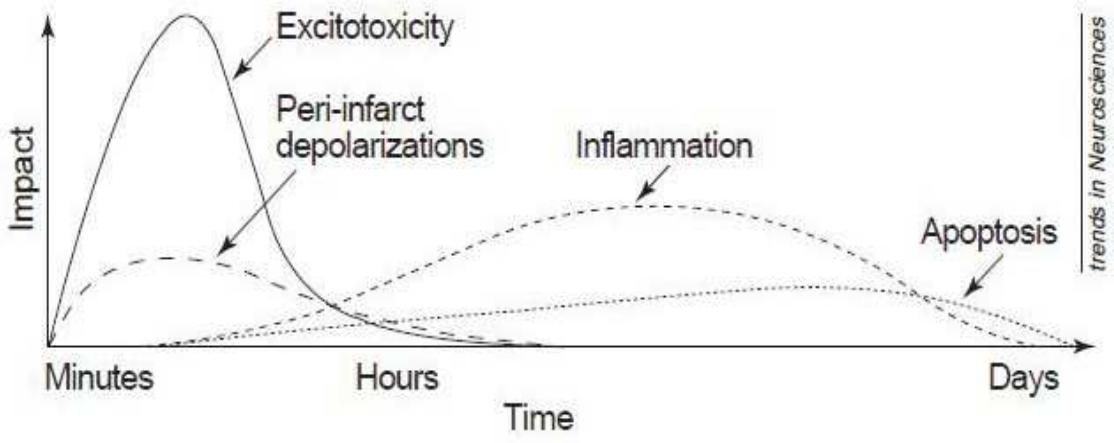


Fig. 1. Cascade of damaging events in focal cerebral ischaemia (Dirnagl et al. 1999).

1.2.1. Phase of excitotoxicity, ionic imbalance and periinfact depolarization

Oxygen and glucose deficit caused by ischemia incident leads to deficiency of adenosine triphosphate (ATP) produced by oxidative phosphorylation in mitochondria. ATP is necessary to maintain and restore ionic gradients, e.g. by Na+/K+ ATPase nessesary for the action potential (Doyle et al. 2008).

Energy failure leads to ionic disbalance and membrane depolarization. It allows the excitatory neurotransmitter glutamate is released and causes increased intracellular Ca^{2+}, Na^{+}, Cl^{-} levels while K^{+} is released into the extracellular space. Release of glutamate and K+ can cause further peri-infarct depolarizations (Doyle et al. 2008). Due to ionic disbalance, water shifts to the intracellular space via osmotic gradients which may result in oedema affecting the perfusion of surrounding tissue and resulting in increased intracranial pressure, vascular compression and herniation (Dirnagl et al. 1999). Glutamate also activates N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Opening of NMDA channels and changing in a subunit of AMPA channels, leads to further membrane depolarization and greater calcium influx, exacerbating intracellular calcium overload (excitotoxicity) (Doyle et al. 2008).

Calcium, the universal intracellular messenger, overactivates many catabolic enzymes such as calcium dependent proteases, lipases and DNAses and it leads to death of many cells in the ischemic core. Among other enzymes, calcium triggers neuronal nitric-oxide synthese (NOS) that synthesize nitric oxide (NO). It reacts with a superoxide anion and form highly reactive peroxynitrite, a potent oxidant (Dirnagl et al. 1999). This production of free radicals and oxidative stress leads to over-activation of poly(ADP-ribose)polymerase-1 (PARP-1), a DNA repair enzyme, which catalyzes the transformation of β-nicotinamide adenine dinucleotide (NAD+) into nicotinamide and long polymers of poly(ADP-ribose). When PARP-1 is over-activated it depletes cells of NAD+, impairing NAD+ dependent processes such as anaerobic glycolysis and mitochondrial respiration, which leads to ATP starvation, energy failure and neuronal death. Oxygen free-radicals also serve as important signaling molecules that trigger inflammation and apoptosis (Dirnagl et al. 1999).

1.2.2. Phase of inflammation

The inflammatory response is a composite process that involves many different cell types, inflammatory mediators and extracellular receptors

The Cellular Inflammatory Response

Stroke causes neutrophilia, lymphocytopenia and an increase in the number of circulating monocytes (Ross et al. 2007) which contributes to ischemic damage.
First cells that accumulate in the brain as early as 30 minutes after permanent middle cerebral artery occlusion (MCAO) are neutrophils. Its recruitment to ischemic brain begins with neutrophil rolling on activated endothelial blood vessel walls, mediated by selectins (e.g. P-selectin, E-selectin) followed by neutrophil activation and adherence, mediated by integrins and immunoglobins. Adhesion to vessel is enabled through a contact between integrins and endothelial intercellular adhesion molecule–1 (ICAM-1). Subsequently, neutrophils transmigrate into the cerebral parenchyma, a process facilitated by blood brain barrier (BBB) disruption (McEver 2001). The recruitment of neutrophils can obstruct the microcirculation which can be followed by the increased severity of ischaemia (Dirnagl et al. 1999). Moreover, neutrophils cause release oxygenfree radicals and proteolytic enzymes causing the tissue damage.
The bone marrow derived cells accumulate in the ischemic brain and become the predominant cell in the late stages of infarct. They bind to vascular cell adhesion molecule (VCAM), transmigrate in the cerebral parenchyma and persist in the ischemic brain for weeks following stroke (Danton and Dietrich 2003).
Microglia/blood derived macrophages play a key role in the development of ischaemic lesions. Althought the initial infiltration leads to worsening of tissue damage and exacerbation of neurological deficits, in the later phases, microglia can phagocytose debris, attack microorganisms that may infect the damaged area and release cytokines that promote glial scar formation (Danton and Dietrich 2003).
Microglia and perivascular cells mediate the intrinsic neuroinflammatory response. Microglia are generally considered to be derived from the monocytic lineage. They reside in a ‘‘resting’’ but vigilant state throughout the parenchyma and express few

surface molecules identifying them as potential phagocytes. Four to six hour after ischemia, microglia/macrophages become activated and migrate to site of injury and asume an ameboid morphology, differentiate, phagocytose and secrete a wide variety of molecules icluding the same markers as peripheral macrophages (Eglitis and Mezey 1997). In this state they are virtually indistinguishable from peripheral macrophages (Danton and Dietrich 2003).

Lymphocytes, ordinarily excluded from the central nervous system (CNS), appear within 24 hours in post ischemic brain (Schroeter et al. 1994). Although the exact mechanism producing their infiltration into the brain remains unclear, BBB disruption plays a major role in the influx, either by directly allowing free lymphocyte movement, or by leakage of brain antigens resulting in the transmigration of activated lymphocytes (Danton and Dietrich 2003). Lymphocytes appear to contribute to development of the lesion in the cortex (Hurn et al. 2007)

The cytokine inflammatory response

Injured brain produces cytokines and chemokines which initiate a localized inflammatory response and contribute to stroke related brain injury.

During ischemia, cytokines, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNFα), transforming growth factor (TGF-β) produced by a variety of activated cell types.

Production of IL-1 is increased in microglia, astrocytes, and neurons. Among other possible deleterious effects of IL-1 (fever, arachidonic acid release, enhancement of NMDA mediated excitotoxicity, and stimulation of nitric oxide synthesis) is the additional role of IL-1 recruitment and adhesion of neutrophils through up-regulation of E-selectin, ICAM-1, ICAM-2, and VCAM-1 on cerebral endothelial cells (Huang et al. 2006).

Expression of IL-6 appears 3hours after occlusion, and it is unclear whether its effect is beneficial or detrimental in the context of stroke. On the one hand, it has proinflammatory role and its biological activity overlaps with those of IL1, however, IL-6 also has antiinflammatory properties due to its ability to induce IL-1 receptor antagonist synthesis (Danton and Dietrich 2003).

Up-regulation of TNF-α has shown to aggravate the ischemic injury and like IL-1, TNF-α promotes neutrophil accumulation and transmigration (Huang et al. 2006). In addition, TNF-α disrupts the blood-brain barrier and stimulates the induction of other inflammatory mediators.

Another cytokine, TGF-β was found to be neuroprotective in the pathogenesis of stroke (Huang et al. 2006).

Besides cytokines, endothelial cells, microglia, neurons, platelets, leukocytes, and fibroblasts injured brain produces chemokines such as cytokine-induced neutrophil chemoattractant (CINC) and monocyte chemoattractant protein (MCP) which attracts neutrophils and monocyte towards their target in ischemic tissue (Huang et al. 2006).

1.2.3. Neuronal apoptosis

Apoptosis or programmed cell death is an active process of self-destruction eliminating the damaged or unnecessary cells. In the ischemic brain it occurs in the area surrounding the core, called penumbra, sustaining milder injury and where ATP is still preserved (Doyle et al. 2008). Apoptosis is preferred in this area because cells undergoing apoptosis minimize damage and disruption to neighboring cells. It is triggered by a wide array of stimuli including oxygen free radicals, death receptor ligation, DNA damage, protease activation and ionic imbalance (Doyle et al. 2008).

Cerebral ischemia triggers two pathways of apoptosis- the intrinsic and extrinsic pathway.

The intrinsic pathway is originated from release of cytochrom c from the outer mitochondrial membrane caused by ionic disbalance and mitochondrial swelling (Broughton et al. 2009). A lot of facilitating factors are included in this pathway- proteins from Bcl-2 family either promote (Bax, Bak Bad, Bim, Bid) or prevent (Bcl-2, Bcl-XL, Bcl-w) pore formation in the outer membrane which promotes the cytochrome c release (Doyle et al. 2008). Release of cytochom c leads to the downstream activation of caspases through activation of an apoptosome complex[apoptosis- activating factor (APAF1) plus pro-caspase 9] in the presence of dATP (Dirnagl et al. 1999). It promotes activation of caspase 3 (Dirnagl et al. 1999), a key mediator in the intrinsic pathway (Broughton et al. 2009) and it initiates the irreversible phases of apoptosis by cleaving homeostatic, cytoskeletal, repair, metabolic, and cell signaling proteins (Doyle et al.

2008). Also further DNA fragmentation is caused by cleaving DNA repair enzymes, such as poly (ADP-ribose) polymerase (PARP) (Broughton et al. 2009) and by activating capase-activated deoxyribonuclease (CAD). Caspase activation can be modulated by protein inhibitors of apoptosis (IAP) and indirectly by secondary mitochondria-derived activator of caspase (Smac/Diablo) (Doyle et al. 2008).

Extrinsic mechanism, also referred to as the death receptor pathway, originates from the activation of cell surface death receptors which belong to the necrosis factor receptor (TNFR) superfamily. This includes many receptors, among other Fas receptor. When a ligand of this receptor (FasL) is bound, an adaptor molecule Fas-associated death domain (FADD) protein is bound to procaspase-8 by interacting with its death effector domain. This complex (FasL–Fas–FADD–procaspase-8) is referred to as death-inducing signaling complex (DISC) and releases caspase-8 by the proteolytic cleavage. Subsequently caspase 8 in turn activates caspase-3 and this effector caspase cleaves PARP and activates CAD, leading to DNA damage and cell death as in intrinsic pathway. Caspase-8 can also activate one of the Bcl-2 family proteins, Bid, and initiates the mitochondrial pathway of apoptosis (Sugawara et al. 2004).

1.3. NICOTINIC ACID

Nicotinic acid (niacin; vitamin B3), a member of water soluble vitamins has been used to treat a wide range of lipid disorders and to successfully prevent clinical cardiovascular disease for nearly 50 years (Meyers et al. 2004).

It modifies lipid profiles by lowering total plasma cholesterol, triglycerides (TG*a*), very-low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol (LDL-C) and it increases high-density lipoprotein cholesterol (HDL-C) (Carlson 2005). Nicotinic acid (NA) treatment has also been shown to slow the progression of atherosclerosis and significantly reduce coronary disease morbidity and mortality when taken alone or in combination with statins (Brown and Zhao 2008) (Canner et al. 1986).

Usage of niacin is limited by its side effect- a strong cutaneous vasodilatation called flushing- which occurs in 80% by patient first administration (Kamanna et al. 2008). It has been reported that niacin flush is mediated by prostaglandin D2 (PGD2) (Morrow et al. 1989) and PGE2 produced by Langerhans' cells in the skin and arachidonic acid released by phospholipase A2 (Benyo et al. 2006). Thus, flushing can be reduced by alteration the pharmacokinetics of the niacin which has been used in the modified release formulation of niacin (McKenney et al. 1994). Also the latest discovered inhibitor of the prostaglandin DP1 receptor, laropiprant successfully reduces flushing by 80% when combined with niacin in a novel formulation niacin- laropiprant (Cheng et al. 2006). However, NA treatment is known to be hepatotoxic (Kamanna et al. 2008) even though the exact mechanisms of this side effect are still unclear.

1.3.1. GPR109A

Recently, the niacin receptor was discovered and was reported as a G-protein-coupled receptor (GPCR) existing in the spleen, adipose, and macrophages (Lorenzen et al. 2001). This receptor is named as GPR109A, also reffered to as HM74 in humans and "protein up-regulated in macrophages by interferon γ (PUMA-G) in mice (Schaub et al. 2001). This receptor was shown to couple to members of the G_i family of G proteins.

In adipocytes, binding of nicotinic acid is reported to inhibit lipolysis by inhibiting adenylyl cyclase resulting in decrease in intracellular cyclic adenosine monophosphate (cAMP) levels (Aktories et al. 1980). It inactivates protein kinase A, which leads to the suppression of hormone-sensitive lipase (Tunaru et al. 2003).

The beneficial effects of NA are not only mediated through alterations in plasma lipid levels but expression of GPR109A was also found in immune cells such as monocytes, macrophages and neutrophils which can be regulated by various cyrtokines. Activation of G_i in immune cells primarily results in the stimulation of β- isoform of phospholipase C or of phosphoinositide-3-kinase γ via G protein βγ-subunits released from activated G_i (Gille et al. 2008). Studies describing the novel anti-inflammatory role of niacin show that niacin decreases monocyte and macrophage adhesion and accumulation in atherogenesis (Gille et al. 2008).

The most recent finding suggests that NA inhibits the upregulation of MCP-1 expression in response to interferon γ (IFNγ) in isolated macrophages as well as in plaque macrophages. Additionaly, nicotinic acid possibly has effect on differentiation of macrophages (Lukasova et al.).

1.4. MACROPHAGES

Monocytes originate in the bone marrow from a common haematopoietic stem cell (HSC). During their development, myeloid progenitor cells (termed granulocyte/macrophage colony-forming units) give rise to monoblasts and then they divide and differentiate into pro-monocytes and finally monocytes, which exit the bone marrow and enter the bloodstream. They transfer into circulating peripheral blood mononuclear cells (PBMcs), which migrate into tissue in the steady state or in response to inflammation. They differentiate in macrophages to replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Gordon and Taylor 2005).

In the blood, monocytes are not a homogeneous population of cells. Two populations of-monocytes have been identified in mice. Inflammatory monocytes rapidly exit the blood and they are defined as GR1+ (also known as Ly6) $CX3CR1^{low}$ (CX3C-chemokine receptor 1). The second group, resident monocytes are defined as GR- $CX3CR1^{hi}$ (Geissmann et al. 2003). It is not clear whether these two cells represent distinct monocyte populations or if inflammatory monocytes mature into resident monocytes (Mosser and Edwards 2008). But human monocytes seem to have distinct physiology from that of mouse monocytes. (Passlick et al. 1989).

Macrophages (MF) differentiate from monocytes and they are phagocytic cells that recyclate iron and hemoglobin from erytrocytes. They are also involved in the removal of cellular debris that is generated during tissue remodelling, and rapidly and efficiently clear cells that have undergone apoptosis**.** MF are essential for host defense- they are one of the primary sensors of danger in the host. The endogenous danger is detected through Toll-like receptors (TLRs), intracellular pattern- recognition receptors, the IL-1R. Majority of them transfers signal through the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88) (Mosser and Edwards 2008).

MF have remarkable plasticity that allows them to efficiently change their phenotype, and their physiology can be markedly altered by both innate and adaptive immune responses. MF have been classified according to T- cell literature, where M1 MF represent one extreme and M2 MF represent another depending on the cytokine

response that they induce. M1 MF represent classically activated MF and M2 alternative activated MF (Gratchev et al. 2001; Mosser and Edwards 2008). The response of M1/M2 MF can influence whether Th1/Th2 types of inflammatory responses occur (Mills et al. 2000).

Althought monocyte and MF heterogenity and nomenclature are subject of discussion (Geissmann et al.; Gabrilovich and Nagaraj 2009), M2 designation encompasses cells with dramatic differences in their biochemistry and physiology. Hence, new grouping of MF populations was described based on three different homeostatic activities — host defence, wound healing and immune regulation (Mosser and Edwards 2008).

1.4.1. Classically activated macrophages

Classically activated MF represent products of a cell- mediated immune response with microbicidal or tumoricidal activity.

They arise in response to adaptive or innate immune signal. During innate immune response natural killer (NK) cells produce interferon-γ that is also a product of TH cells or CD8+ cells during adaptive immune response. Additionally, classically activated MF can be activated by tumor necrosis factor, a product of antigen-presenting cells (APC), which is induced by a TLR ligand action in a MyD88- dependent manner (Mosser and Edwards 2008).

Ligation of TLR or TNF receptor may activate nuclear factor-κB and mitogen-activated protein kinases, IFNγ receptor ligation activates signal transducer and activator of transcription molecules. Combination of all these transcription factors triggers all genes involved in the activation of classically activated MF (O'Shea and Murray 2008).

In answer to IFNγ and TNF signal, classically activated MF can secrete pro-inflammatory cytokines, produce increased amounts of superoxide anions and oxygen and nitrogen radicals to increase their killing ability (Dale et al. 2008). The pro-inflammatory cytokines produced by classically activated macrophages are for example, IL-1, IL-6 and IL-23. They have been associated with the development and expansion of Th17 cells, which can contribute to autoimmune responses (Bettelli et al. 2006). Classically activated MF can also produce IL-12 to promote the differentiation of Th1 cells, on the other hand they can also produce IL-27, which inhibits various immune responses and negatively regulates Th1 and Th2 cells.

Cytokines and mediators produced by classically activated MF can also lead to host-tissue damage, therefore their activation must be tightly controlled (Mosser and Edwards 2008).

1.4.2. Wound-healing macrophages

Wound healing MF can develop also in response to innate or adaptive signals. They are generated in presence of IL-4 which can be produced by Th2 cells or by granulocytes (Brandt et al. 2000). These macrophages are poor APC and may inhibit T-cell proliferation and they produce minimal amount of pro-inflammatory cytokines.
Their primary function seems to be related to wound healing due to their ability secrete components of the extracellular matrix (Mosser and Edwards 2008).

1.4.3. Regulatory macrophages

Regulatory MF are generated in response to various stimuli, including immune complexes, prostaglandins, GPCR ligands, glucocorticoids, apoptotic cells or IL-10, produced by regulatory T cells (Mosser and Edwards 2008).
The most important and reliable characteristic of regulatory MF is their anti-inflammatory activity. To induce this activity, two stimuli are needed, while the first signal has little or no stimulatory function on its own. However, when a second stimulus is added, such as a TLR ligand, macrophages start to produce IL-10 (Edwards et al. 2006), which suppress immune response by inhibition of production and activity of various pro-inflammatory cytokines and it can induce the expansion of Th2 cells. In addition to IL-10 production, these regulatory MF also downregulate IL-12 production; Due to IL-10 production, regulatory MF are inhibitors of inflammation, while they are able to produce many pro-inflammatory cytokines (Mosser and Edwards 2008).
In addition, variety of these regulatory macrophages expresses high levels of co-stimulatory molecules (CD80 and CD86) and therefore can act as APC.

2. MATERIALS

2.1. Equipment and Instrument

Operation microscope SM33	Hund, Wetzlar, GE
Bipolar tweezers	Erbe, Tübingen, GE
Centrifuge 5415R	Eppendorf, GE
Cold light source FLQ 150	Hund and Wetzlar, GE
Cover glass 24x60 mm	ROTH Karlsruhe, GE
Cryotome Leica CM3050S	Leica, Mannheim, GE
Dissection scissor	FST, Heidelberg, GE
FACSCalibur	Becton Dickinson
Fat pen	Dako, Dennmark
Hippocampus spoon	FST, Heidelberg, GE
Micro liter syringes Hamilton	FST, Heidelberg GE
Microtome blades S35	Feather, Japan
Microtube 1,5 ml	Sarstedt, Nümbrecht, GE
Minimot drill mashine 40/E	Proxxon, Niersbach, GE
Mini-Vannas iris scissor	FST, Heidelberg, GE
Needle holder	FST, Heidelberg, GE
Pasteur pipette	Neolab, Heidelberg, GE
Pipette	Geiner Bio-One, Frickenhausen, GE
Pipette tips	Geiner Bio-One, Frickenhausen, GE
Polysine slides	Thermo Scientific, Braunschweig, GE
Small scissors	FST, Heidelberg, GE
Staining dish	Neolab, Heidelberg, GE
Sterile 15ml Falcons	Sarstedt, Hildesheim, GE
Tissue freezing medium	Jung,Nussloch, GE
Tweezers size 6	FST, Heidelberg, GE

2.2. Chemicals and Comercial Kit

2, 2, 2-Tribromoethanol	Fluka chemicals, Seelze, GE
2-Methyl-2-butanol	Fluka chemicals, Seelze, GE
Acetone	Merck, Darmstadt,GE
Ammonia solution 25%	Merck, Darmstadt, GE
Bovine serum albumin (BSA)	SIGMA-aldrich Co., USA
DAB substrate kit for peroxidase	Vector Laboratories, Burlingame, USA
Diphteria Toxin powder	SIGMA-Aldrich Co., USA
Distilled water	B Braun, GE
Formaldehyde 37%	Merck, Darmstadt, GE
Hydrogen Peroxide	CARL ROTH, Karlsruhe, GE
Hydroquinone	Fluka chemicals, GE
Kalium chloride	Applichem, Darmstadt, GE
Lithium carbonate	Riedel-de Haen, Seelze, GE
Methanol 100%	J.T.Baker, Deventer, NL
Mowiol 4-88	Carl Roth, Karlsruhe, GE
Natrium Chloride	J.T.Baker, Deventer, Holland
Paraformaldehyde (PFA)	Riedel-de-Haën Seelze, GE
Silver nitrate	Carlroth, Karlsruhe,GE
Skin disinfectant (Kodan)	Schülke & Mayer GmbH Nordstedt, GE
Tri-Sodium citrate	Sigma-Aldrich, Steinheim
Triton X-100	Merk, Darmstadt, GE
Tween 20	Carl Roth, Karlsruhe, GE
Type F Immersion liquid	Leica, Mannheim, GE

2.3. Antibodies

CD11b rat anti mouse antigen, Serotec, Cat. No. MCA711G
Alexa Fluor 488 donkey anti rat IgG (H+L), Invitrogen, USA, Cat. No. 538955
DAPI (4‘,6‘- diamidino-2-phenylindole), Sigma, Germany

2.4. Computer hardware and software

Scanner Canon 8600F
Fluorescence Microscope, Leica DM4000B
Image J
PictureFrame(TM) Application 2.3
Microsoft Office

3. METHODS

3.1. METHODS FOR ANIMAL EXPERIMENT

3.1.1. Experimental Animals

In our experiments 8-12 weeks old C57BL/6 mice (wild type mice) and transgenic CD11bDTR (Duffield et al. 2005) mice were used.

3.1.2. Anaesthesia and infarct model

Mice were anaesthetised by intraperitoneal injection of 150 µl 2.5% avertin per 10 g body weight. To confirm whether mice were sufficiently anesthetized paw-reflex was tested. The skin was disinfected at the site of incision. A skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed by electrical coagulation. The stem of the middle cerebral artery (MCA) was exposed through a burr hole and was occluded by micro bipolar coagulation tweezers. The wound was continuously washed with isotonic saline during the procedure of removing the temporal muscle and temporal bone to avoid the tissue damage due to heat produced by coagulation. Surgery was performed under a microscope. Mice were kept at a body temperature of 37 °C on a heating pad. The skin was stitched with continuous sutures.

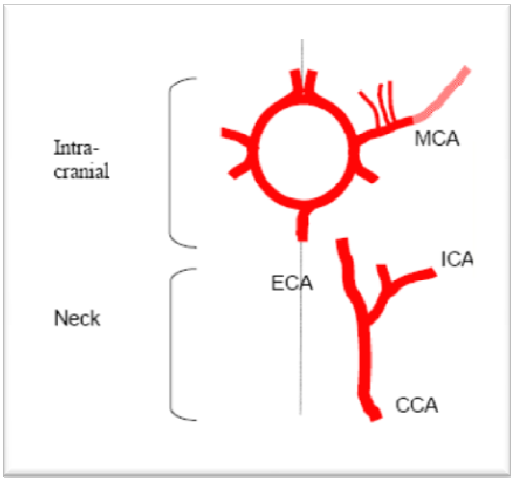


Fig. 2. Model of permanent middle cerebral artery occlusion.
CCA: Common carotid artery; ECA: External carotid artery; ICA: Internal carotid artery; MCA: Middle cerebral artery

3.1.3. Blood sampling from the mice

Mice were anaesthetised as explained above. The skin was disinfected and abdominal cavity was opened. The intestine was gently placed to the left side of the mouse in the abdominal cavity to expose the inferior caval vein. Blood probes were taken from abdominal caval vein with 1 ml syringe.

3.1.4. Perfusion of the brain

The mice were deeply anaesthetised with avertin and fixed on a cork plate. The mice were disinfected on the ventral side. Median thoracotomy was carried out to expose the heart. Intracardial perfusion was performed using 20 ml Ringer's solution for each mouse. The perfusion was monitored by change in colour of the liver that turns yellow during perfusion. The head was cut at the atlantooccipital joint and the brain was removed carefully from the skull with the help of a hippocampus spoon and immediately frozen on dry ice.

3.2. METHODS FOR INFARCT MEASUREMENT

3.2.1. Histological slide

The cryomicrotom was set to a temperature of -25 °C and frozen brains were placed in the microtom chamber to bring them to the same temperature before fixing. The brain was fixed with the help of tissue adhesive (tissue freezing medium Jung®). The coronal sections were cut with a thickness of 20 µm after each 400 µm, starting from rostral to caudal. The sections were placed on adhesion slides.

3.2.2. Silver staining

Silver impregnation solution was made by mixing 67.5 ml saturated (0.6 g in 50 ml H_2O) lithium carbonate solution to 33.75 ml 10% silver nitrate solution. The precipitates formed from the solution were titrated by drop-wise adding 25% ammonia solution (approximately 3.6 ml). The addition of the ammonia solution is a critical step because the excesss of ammonia makes the staining faint but a little precipitate probably does not harm. Finally, 506.25 ml distilled water was added to the prepared solution. The solution was protected from the light.

The developing solution was prepared by dissolving 6.6 g sodium citrate in 420 ml distilled water. Subsequently, a filtered solution of formaldehyde (120 ml 37%) was added and mixed well. Finally, 1.8 g hydroquinone and 90 ml acetone were added. The solution was mixed well at room temperature for around 30 to 60 minutes until the colour changed from yellow to orange. Both solutions were used only once for each 30 slides and the staining was performed according to the following protocol (Vogel et al. 1999).

• The sections were incubated 2 minutes in silver impregnation solution and shacked continuously.

• After the incubation the slides were washed in distilled water 6 times for 1 minute each time.

• Finally the slides were incubated for 3 minutes in developing solution followed by 3 washes in distilled water, each for one minute.

The sections were air dried overnight.

3.2.3. Scanning and infarct measurement

Stained sections were scanned at 300 dpi and the infarct area was measured using Image J software. The data were exported in Microsoft Excel. The silver staining represents not only the infarct area but also surrounding brain oedema as white area. Oedema was corrected as described (Swanson et al. 1990). Y=U-N+I. Where

Y = Corrected infarct (mm^3)

U = Total area of non infarcted hemisphere (mm^3)

N = Total area of infarcted hemisphere (mm^3)

I = Direct measured infarct area (mm^3).

3.2.4. Statistical Analysis

Data are illustrated as mean ± SEM. Statistical comparisons of three or more groups were made by analysis of variance (ANOVA) followed post test by Newman-Keuls Multiple Comparison Test. Two groups were compared by a two-tailed t-test with Bonferroni correction. Values are considered to be significant at $p<0.05$.

3.3. IMUNOLOGICAL METHODS

3.3.1. Microglial staining

First, the phosphate buffered saline (PBS) 1x solution was prepared. It was mixed 8.0 g NaCl, 0.2 g KCl, 1.8 g $Na_2HPO_4.2H_2O$ and 0.24 g KH_2PO_4 and adjusted to pH 7.4 with HCl. Then distilled H_2O was added up 1000 ml.

Frozen sections were air dried for 5 minutes and then fixed with 4% PFA for 25 min at room temperature. The sections were rinsed three times with 1xPBS for 5 minutes each. Then the sections were permeabilized by incubating twice with 0.025% Triton X-100 in 1xPBS for 5 minutes each. Then the sections were blocked with 1% BSA in PBS for 90 minutes at 4°C. The sections were washed three times with PBS for 5 minutes each and were incubated over night at 4°C in a humid chamber with primary antibody (CD11b, Rat anti-mouse antibody, Serotec Cat. No. MCA711G). The primary antibody was diluted 1:100 in 5% BSA. On the next day, the sections were washed three times with PBS for 5 minutes each. The secondary antibody (Donkey anti rat alexa 488, Molecular Probes, Cat.No. 538955) was diluted 1:200 in 5% BSA and added for 1 hour at room temperature. The sections were washed three times with PBS for 5 minutes each and then the sections were incubated with DAPI solution (1:5000 in water) for 10 minutes. The sections were washed three times with water for 2 minutes and then mounted with vector permanent mounting medium and dried for 1 hour.

3.3.2. Fluorescence- activated cell sorting

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry, which is a technique for counting and examining microscopic particles, such as cells and chromosomes. FACS is based upon the specific light scattering and fluorescent charateristics of each cell. All FACS in our experiment were provided by Dr. med. S. Muhammad.

4. RESULTS

4.1. Depletion of CD11b positive cells in CD11bDTR mice

To investigate whether the effect of nicotinic acid is mediated by CD11b positive cells, the CD11bDTR transgenic mice were used in combination with diphtheria toxin (DT) treatment.

This mouse strain expresses the human diphtheria toxin receptor (hDTR) from a monocyte/macrophage-specific CD11b promoter sequence so administration of DT selectively kills monocytes/macrophages (Duffield et al. 2005).

In our case for selective depletion of CD11b positive cells, we injected diphtheria toxin (ip, 25 ng/g body weight) to both wild type and CD11bDTR mice for two consecutive days and then blood samples were taken for FACS analysis.. We found 92% reduction of CD11b positive cells in CD11bDTR mice when compared with the corresponding control mice. (Fig. 3)

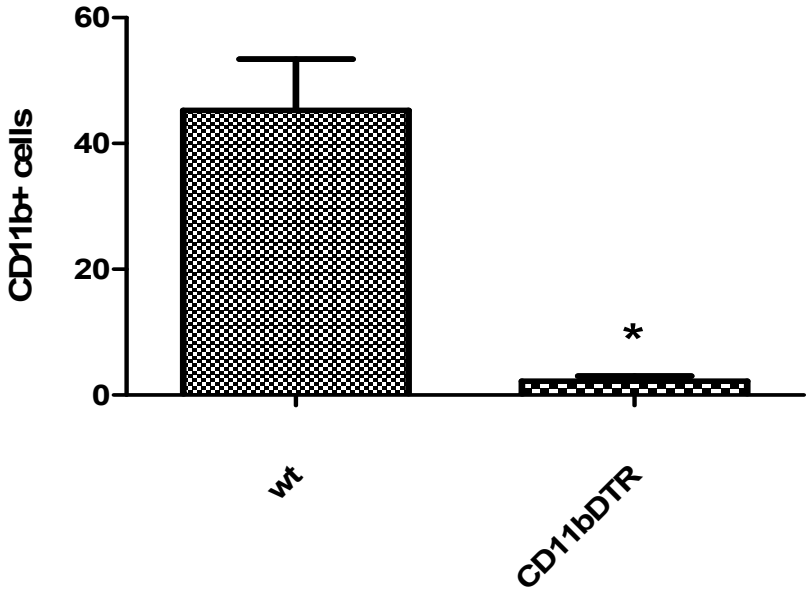


Fig. 3**. Depletion of CD11b$^+$ cells in CD11bDTR mice after DT treatment**

Diphtheria toxin (ip, 25 ng/g body weight) was injected to wt or CD11bDTR mice 48 h and 24 h before analysis of blood cells. To count the cells, a FACS analysis was used and 92% of CD11b+ cells were reduced in CD11bDTR mice.

Data are presented as mean ± SEM; * p=0.003 (t-test).

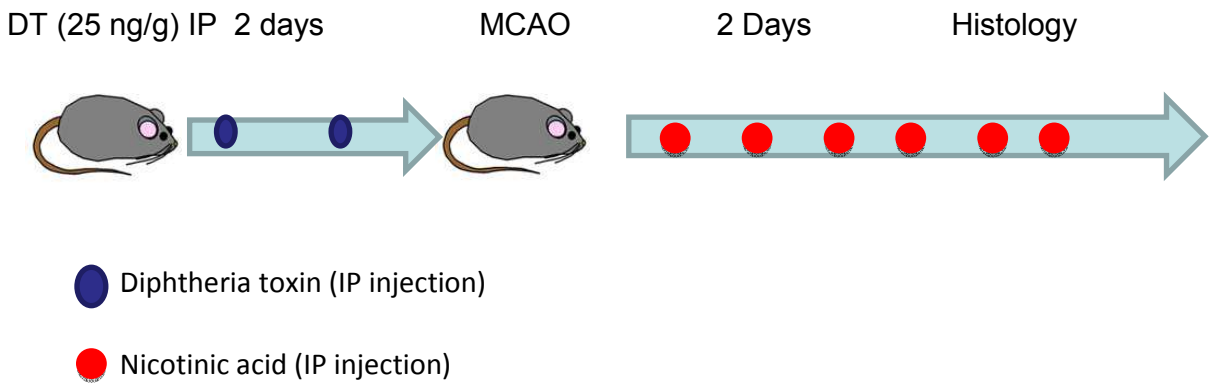


Fig. 4. **Visualisation of Diphtheria toxin treatment and Nicotinic acid treatment** Diphtheria toxin (IP, 25 ng/g body weight) was injected to WT or CD11bDTR mice 48 h and 24 h before MCAO. Mice were treated with either vehicle (NaCl) or nicotinic acid (100 mg/kg, three times a day) for two days and than euthanized.

4.2. CD11b depletion after DT treatment

In order to analyze the impact of nicotinic acid on CD11b positive cells survival, we sought to quantify the amount of CD11b positive cells in predefined parts of brains of CD11bDTR and control mice after NA/vehicle treatment. As CD11b is a selective marker for monocyte macrophages and microglia, we used immunohistochemistry to label the cells.

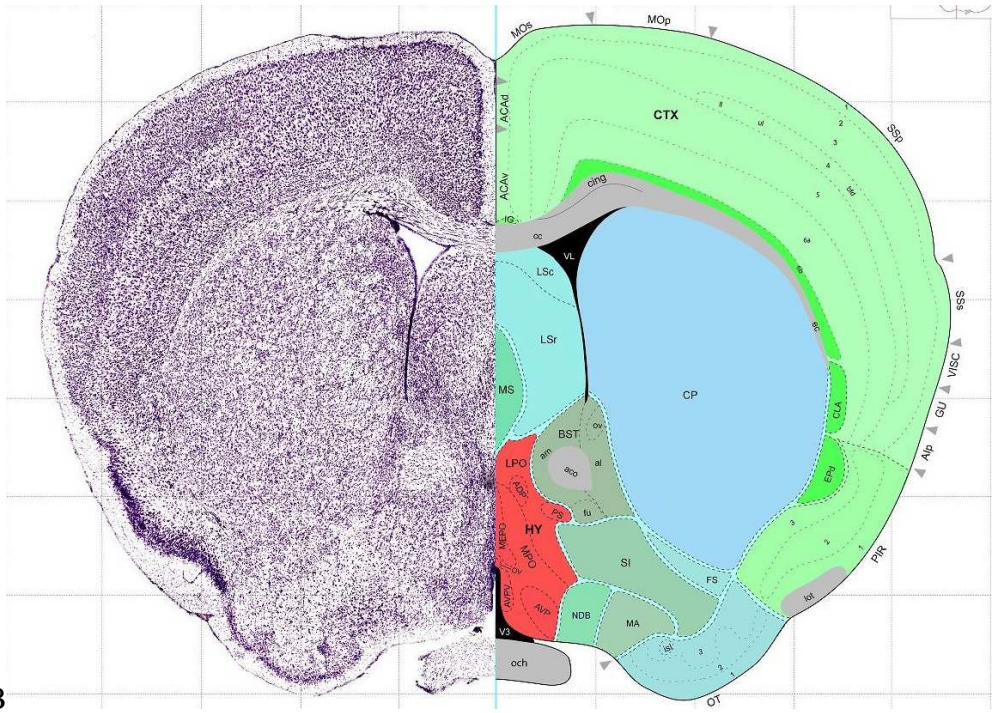
All mice were treated with DT and MCAO was performed. (Fig.4)

For imaging fluorescent microscope (Leica DM4000B) was used. A reference sections from brain-atlas were taken and compared to cryosections of test mice. (Fig.5) Sections No. 25,27,29,31 referred to Paxinos Brain Atlas were used.

For counting the CD11b+ cells 4 brains per group of mice and 4 sections from each brain were used. Quantification was performed in two predefined cortex parts of the ischemic hemisphere of each chosen brain section. (Fig. 5B)

Mean of counted CD11b positive cells in a determined area among all four groups was compared. A significant difference in the number of CD11b positive cells was found in the WT control groups compared to others. No difference was observed in the numbers of CD11b positive cells among other groups. (Fig.7)

A



B

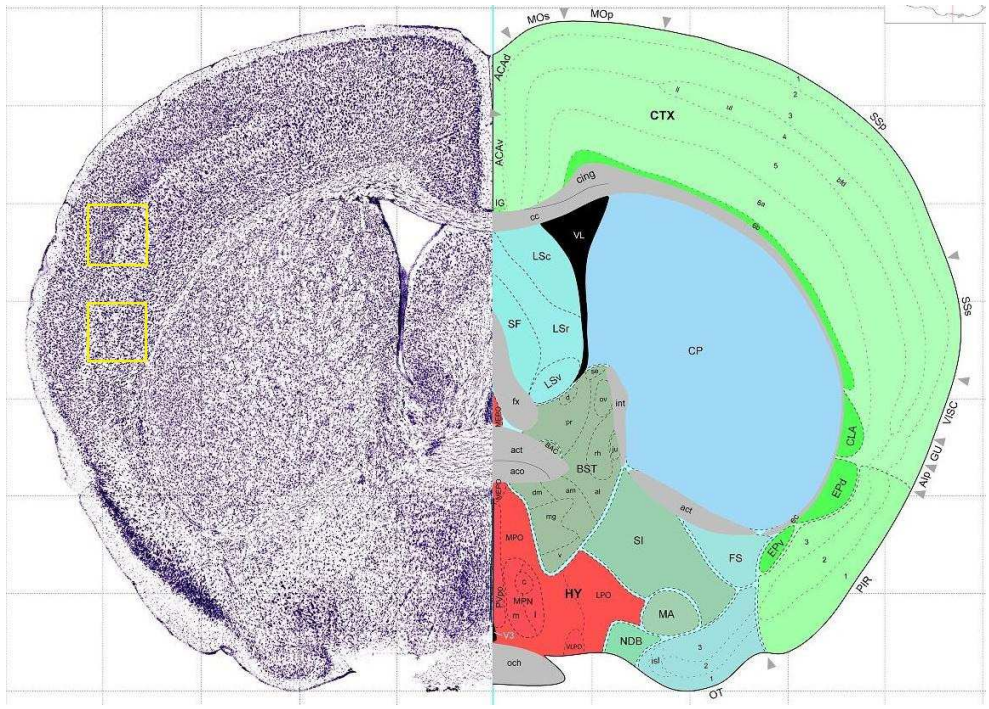


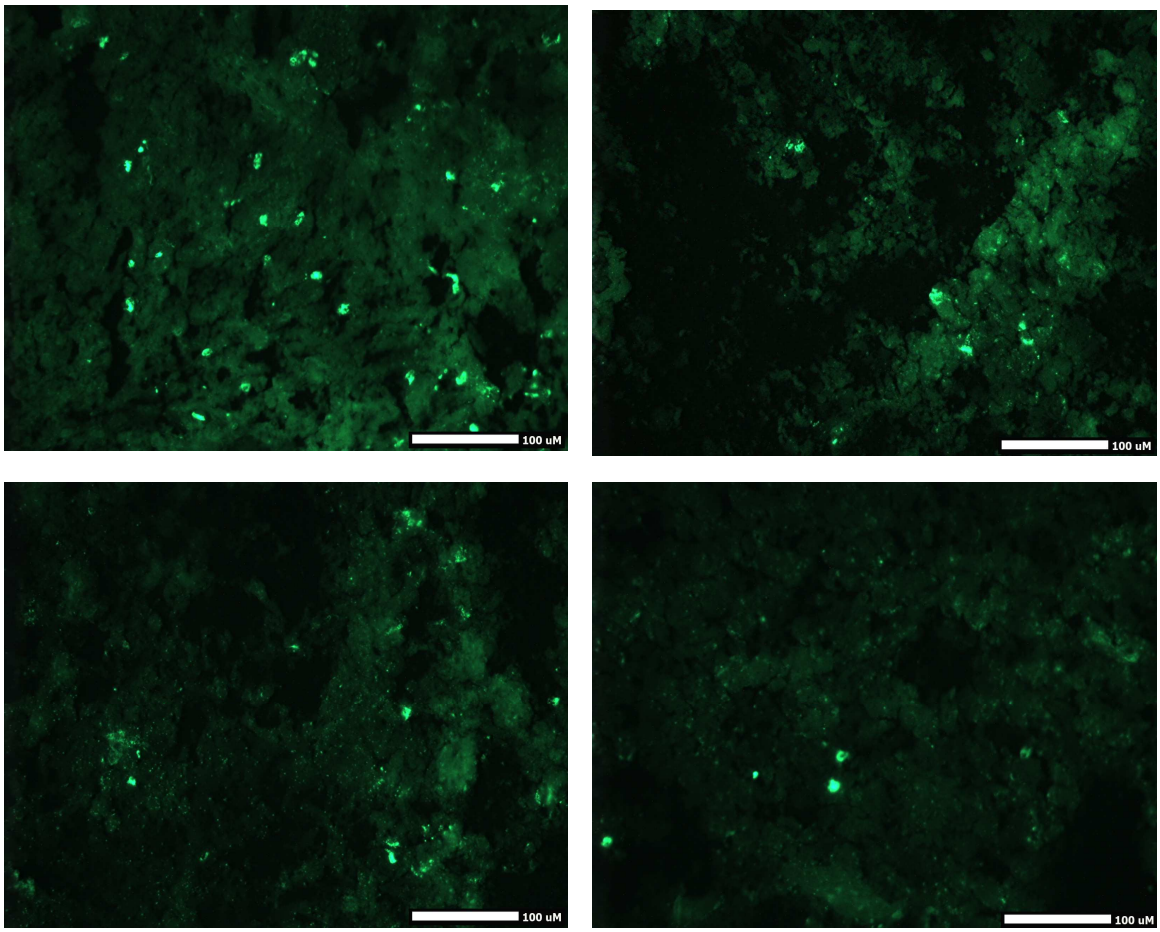
Fig. 5: **Example of brain sections for counting CD11b cells of the cortex**

(A) Section No. 29 reffered to Paxinos Brain Atlas (Bregma 0.26mm)

(B) Section No. 31 reffered to Paxinos Brain Atlas (Bregma 0.02mm)

Yellow borders label places where microscope pictures were taken in each section.

A B



C D

Fig. 6: **Illustration of brain pictures of CD11b+ cells in ischemic area**. Magnifications 20X. Pictures are taken from fluorescent microscope from different mice groups after DT treatment and NA or vehicle treatment and CD11b immunostaining. CD11b+ cells presented as green signals were counted manually by Image J.

(A) WT mice treated with vehicle (B) WT mice treated with NA

(C) CD11bDTR mice treated with vehicle (D) CD11bDTR mice treated with NA

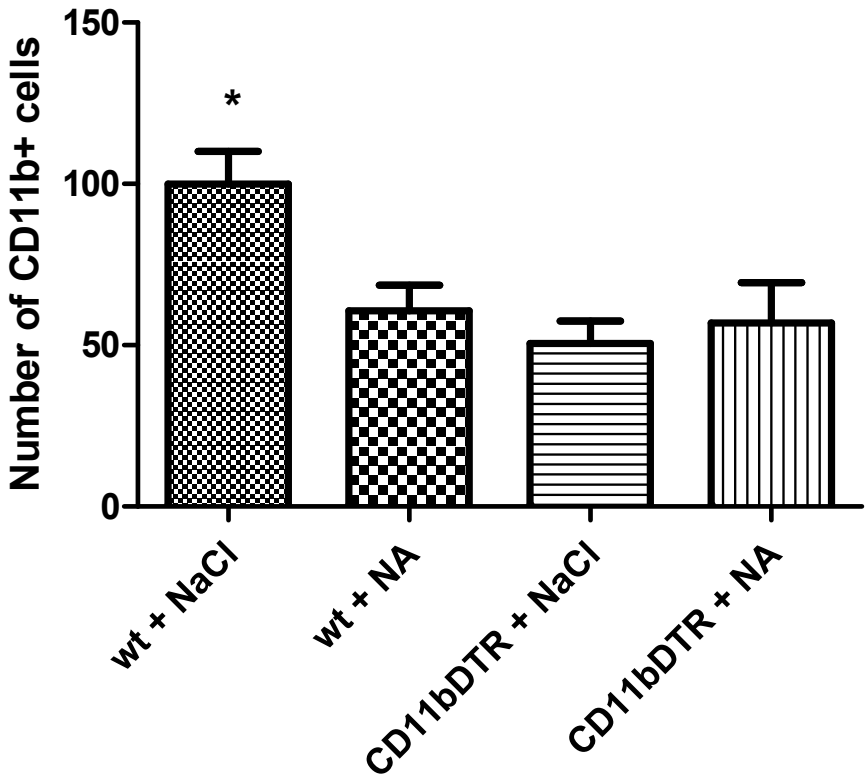


Fig. 7: **CD11b depletion in the ischemic area of the brain after DT treatment**
Mean values of counted CD11b positive cells in predefined area in the ischemic area of the brain after immunohistochemistry with CD11b antigen.(1:100) First the fluorescent pictures were taken and cells were counted manually by using Image J. (n= 4 brains per group, 8 pictures per brain). CD11b+ cells are depleted after NA treatment in WT mice, in between CD11bDTR groups is no influence of NA treatment. Statistic was performed using ANOVA and Newman-Keuls Multiple Comparison Test.
* $p<0.05$ WT+NaCl group compared to all other groups

4.3. Depletion of blood derived CD11b+ cells leads to loss of protection by nicotinic acid

In order to analyze the impact of NA on blood derived CD11b+ cells in cerebral ischemia, the infarct volume was measured. There was found significant difference in infarct volume in the NA treated control group but there was no significant difference in infarct volume in CD11bDTR mice group. To measure the infarct size silver staining was used, that visualizes the infarct area by darkening the surrounding tissue and lightening the infarct area (Fig.6) Around 8-10 brains per group was measured by using Image J program by using formula described in methods.

After comparing the mean values of the infarct area in between the groups, the infarct area reduction after NA treatment was found only in WT mice. We found no significant area reduction between CD11bDTR treated and untreated group. (Fig.7)

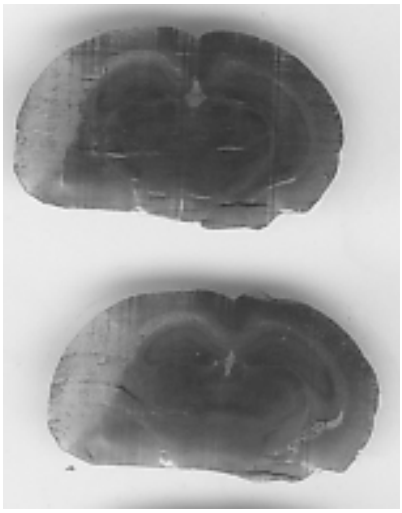


Fig. 8: Samples of scanned brain sections with MCAO after silver staining. Lighter parts of the left hemisphere show the infarct area.

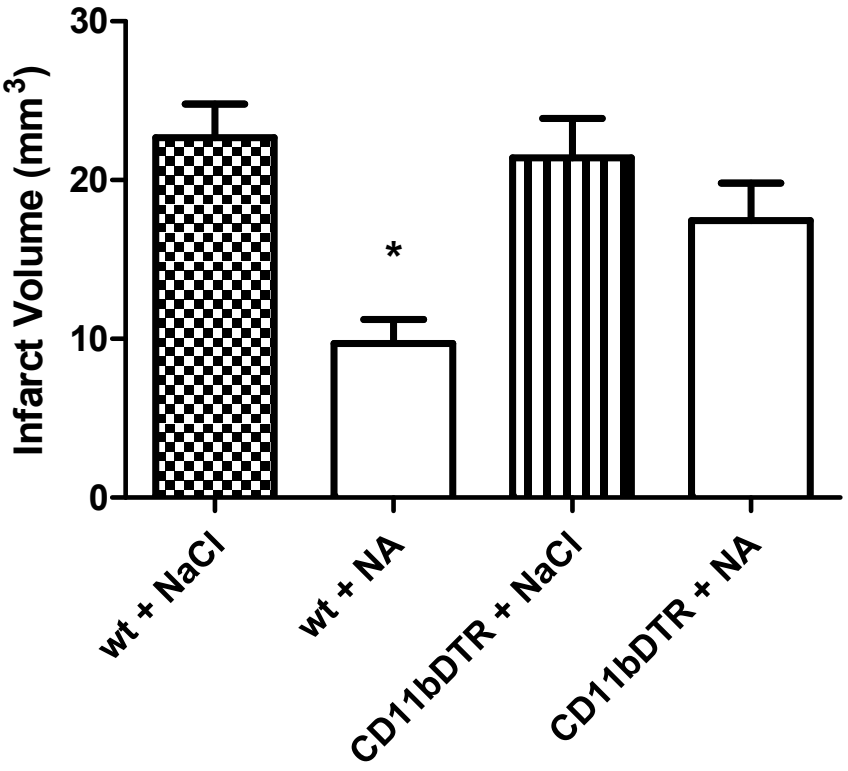


Fig. 9: **Reduction of infarct area in ischemic brain after NA treatment**

Results show significant reduction in the ischemic area only in WT mice treated with NA. It was found no difference between other groups. It demonstrates the protective effect of nicotinic acid only in WT mice. It shows no protective effect of NA on infarct area if the number of CD11b+ cells is reduced as it is in CD11bDTR mice.

5. DISCUSSION

Stroke is one of the leading causes of death worldwide and its effective protective treatment is still missing. Nicotinic acid is widely used as a drug to influence the lipid spectrum and it is successfully used for prevention of cardiovascular diseases (Kamanna et al. 2008). Many studies have been done to determine its effect in atherosclerosis. Recently its receptor GPR109A was found and it mediates the effect of NA including its adverse effect- flushing. This receptor is located among other in adipose tissue and some immune cells including macrophages (Lorenzen et al. 2001).

This study was designed to clarify the role of NA in the cerebral ischemia. To investigate whether macrophages are involved in its effect, we used WT and transgenic mice. Recently developed CD11bDTR mice have been succesfully used for specific depletion of CD11b+ cells (Duffield et al. 2005; Stoneman et al. 2007). Normally, the DTR receptor in mice bind DT poorly compared with the human and simian molecules (Saito et al. 2001), in CD11bDTR mice with transgenic expression of the human DT receptor (DTR) administration of DT selectively kills monocytes/macrophages in blood and some tissues (Stoneman et al. 2007). We treated these transgenic mice with DT and found 92% depletion of CD11b+ cells in their blood (Fig.3) which confirms previous findings.

Macrophages and microglia play an important role in inflammatory phase of cerebral ischemia. In our work we sought to determine the impact of NA treatment on infiltration of macrophages after cerebral ischemia in normal WT mice and compare it with CD11b depleted mice (CD11bDTR). Since CD11b is a marker for macrophages and activated microglia (Rosen and Gordon 1990; Henkel et al. 2004), we stained brain sections with anti- CD11b antibody.

There seems to be found that nicotinic acid treatment reduces the number of CD11b positive cells in the ischemic area of the brain of WT mice (Fig. 7) and in our experiment NA had no signifficant effect on the reduction of CD11b+ cells in CD11bDTR group when blood derived CD11b+ cells were already depleted.

It confirms previous finding that macrophages express GPR109A receptor which mediates the effect of NA (Lorenzen et al. 2002).
However, in cerebral ischemia, brain macrophages consist partly of activated microglia and partly of immigrant macrophages. From our experiment it is not clear how many percent of CD11b+ cells depleted by NA treatment are microglia and how many percent are blood derived macrophages. According to CD11bDTR groups where we found no further reduction between treated and control group, it seems that NA has no effect on the number of microglia in our determined area of ischemic part of the brain. On the other hand, we don't know have sufficient data for the impact of DT on the number of tissue microglia in the brain.
To evaluate the impact of NA treatment on the number of activated microglia next experiments must be performed. It could be useful also to determine whereas NA treatment influences only activated microglia and if microglia in contralateral hemisphere are affected, too. Activation of microglia is a hallmark of neuroinflammation that worsens the damage in the post ischemic brain (Gehrmann et al. 1992). Anyway, the only definite way to distinguish between the two populations of macrophages and microglia is transplantation of labeled bone marrow.

This study also provides knowledge about the role of the NA in the infarct area in post-ischemic mice brain. In our experiment, NA reduced the infarct volume significantly in WT group. Surprisingly, the infarct area among other three groups (WT untreated and both CD11bDTR groups) was not significantly changed (Fig.9). It seems that depletion of blood derived CD11b + cells leads to loss of protection of NA treatment.

The most recent finding, made by Lukasova et al., shows that NA has a potential to change the differentiation state of MF between M1 and M2. In their study concerning atherosclerosis they explored that NA induces reduced arginase-2, a marker for M1 macrophages (Lukasova et al.).
In our lab we tried to perform immunohistochemistry with arginase-1 as a marker for M2 macrophages and iNOS as a marker for M1 (Hesse et al. 2001) but the exact staining protocol must be developed first.

In conclusion, from our experiments nicotinic acid seems to have a protective effect in ischemic brain which could be mediated by macrophages but to verify this effect and to find exact mechanism further research is needed.

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